SYMBIOSIS BETWEEN CHLORELLA SP. AND AZOTOBACTER CHROOCOCCUM AND NITROGEN FIXATION.

By C. B. LIPMAN and L. J. H. TEAKLE.

(*From the Plant Nutrition Laboratory, University of California, Berkeley.*)

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Alfred Koch has given considerable attention to the subject of nitrogen fixation by bacteria as resulting from their symbiosis with algæ. Many others have also investigated various features of this problem. In spite of all this there are numerous questions which remain unsolved. The use of soil cultures for such studies is objectionable on account of the soil's complexity, its variety of flora and fauna, and the impossibility of observing the organisms while they are at work; and yet the soil culture method has been the one most used by investigators heretofore.

A fortunate accident threw into our hands a species of *Chlorella* which we are using in some physiological experiments. The facile growth of this organism under ordinary laboratory conditions in tap water, in distilled water, and in weak culture solutions offered a ready means for experimentation on the question of a possible symbiosis with *Azotobacter*. This strain of *Chlorella* develops in solution cultures a beautiful green granular growth and with proper light conditions covers the walls of the culture flask completely. It is a single celled organism, somewhat elliptical in shape, and not much more than twice as large as the large cells of *Azotobacter chroococcum*. Its cell wall and chloroplasts are very distinct and the structure of the cell unusually clear under the microscope. The organism probably comes from the tap water of the city of Berkeley, but it is undoubtedly a common inhabitant of surface soils. Some unusually striking physiological peculiarities of this strain of *Chlorella* will be described in a later publication from this laboratory.

The *Azotobacter* culture used in this investigation was a pure culture of *Azotobacter chroococcum* (Madera strain) which was isolated from a California soil.

Technique and Results of the Experiment.

A culture of *Chlorella* which had been growing under greenhouse conditions for a number of weeks in an inorganic culture solution in an Erlenmeyer flask stoppered with cotton was transferred to a large graduated cylinder and made up with sterile tap water to a volume of 600 cc. After thorough agitation 50 cc. portions of this diluted culture were measured out into 300 cc. Erlenmeyer flasks. This great dilution of the culture of *Chlorella* should make the total amount of dissolved matter comparable to that of the tap water of the city of Berkeley. This tap water has a total concentration of inorganic salts of approximately 400 parts per 1,000,000. It contains about 65 parts per 1,000,000 of Ca and Mg combined, and about 90 parts per 1,000,000 of Na. The total loss on ignition in the residue of this tap water is about 100 parts per 1,000,000. The organic matter in the culture solution made up as described above would come from the small amount dissolved in the tap water and from the dead cells of *Chlorella* which had accumulated in the original culture. This must, of course, constitute a very slight source of organic matter. *Azotobacter chroococcum* alone, without the presence of living cells of *Chlorella*, in such a culture solution can fix practically no nitrogen. The question, therefore, remains whether it can fix nitrogen when it has with it living cells of *Chlorella* in the same culture solution. No carbohydrate was added to any of the culture solutions used in this experiment.

To every 50 cc. culture prepared as above described 1 cc. of a suspension of a fresh culture of *Azotobacter chroococcum* was added. Six of the mixed cultures were analyzed immediately for total nitrogen by the modified Gunning method. The other six cultures were allowed to incubate in the laboratory in fairly subdued light for a period of a month. The flasks were plugged with cotton to keep out other organisms and dust. In addition to the mixed cultures of *Chlorella* and *Azotobacter* we prepared several similar 50 cc. cultures of *Chlorella* alone which were incubated side by side with the mixed cultures to serve as a control. After a few days of incubation we noticed a marked difference between the *Chlorella* cultures with *Azotobacter* and those without *Azotobacter*. The pure culture of *Chlorella* while normal in appearance was light green in color and seemed to be growing slowly. The mixed culture, on the other hand, was beautiful emerald green and

was growing rapidly, producing a characteristic granular growth. The discrepancy between the cultures of the *Chlorella* alone and those of *Chlorella* with *Azotobacter* was maintained and even grew more marked as the period of incubation progressed. After about 1 month incubation the cultures were analyzed for total nitrogen with the results indicated in Table I.

The data in Table I show clearly that while the absolute gains of nitrogen in the mixed cultures of *Chlorella* and *Azotobacter* were small, the relative gains are large. Moreover, they show that *Azotobacter* is able to use the carbohydrate synthesized by *Chlorella* as a source of energy for nitrogen fixation, since every culture gained in nitrogen. The highest figure for total nitrogen in the six control cultures is

TABLE I.

Total N in Cultures of Chlorella with Azotobacter.

Culture No.	N found.	N in control.	N fixed.
	mg.	*mg.*	*mg.*
1	1.40	0.70	0.70
2	1.26	0.98	0.28
3	1.54	0.98	0.56
4	1.12	0.84	0.28
5	1.26	0.64	0.62
6	1.26	0.64	0.62
Average.....	1.31	0.80	0.51

appreciably lower than the lowest figure for the incubated mixed cultures. When one remembers in addition how small the amount of dry matter is in every culture it is remarkable that on the average about 0.5 mg. of nitrogen was fixed. This indicates a high efficiency of the process under the conditions in question.

Our results in this experiment are preliminary in nature. We hope to carry on futher experiments along this line to discover more about the process of nitrogen fixation in the symbiotic relationship into which *Chlorella* and *Azotobacter* seem to enter so effectively. It would also seem that attractive possibilities exist for practical applications of these studies. In the case of sandy soils particularly it might be possible to inoculate with *Chlorella* and *Azotobacter* to increase the soil nitrogen supply cheaply and effectively.